Microbiological Research 190 (2016) 55–62

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

In *Azospirillum brasilense*, mutations in *flmA* or *flmB* genes affect polar flagellum assembly, surface polysaccharides, and attachment to maize roots

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ARTICLE INFO

Article history:
Received 4 September 2015
Received in revised form 2 April 2016
Accepted 13 May 2016
Available online 14 May 2016

Keywords:
Motility
Root-adsorption
Lipopolysaccharides
Exopolysaccharides

ABSTRACT

Azospirillum brasilense is a soil bacterium capable of promoting plant growth. Several surface components were previously reported to be involved in the attachment of *A. brasilense* to root plants. Among these components are the exopolysaccharide (EPS), lipopolysaccharide (LPS) and the polar flagellum. Flagellin from polar flagellum is glycosylated and it was suggested that genes involved in such a posttranslational modification are the same ones involved in the biosynthesis of sugars present in the *O*-antigen of the LPS. In this work, we report on the characterization of two homologs present in *A. brasilense* Cd, to the well characterized flagellin modification genes, *flmA* and *flmB*, from *Aeromonas caviae*. We show that mutations in either *flmA* or *flmB* genes of *A. brasilense* resulted in non-motile cells due to alterations in the polar flagellum assembly. Moreover, these mutations also affected the capability of *A. brasilense* cells to adsorb to maize roots and to produce LPS and EPS. By generating a mutant containing the polar flagellum affected in their rotation, we show the importance of the bacterial motility for the early colonization of maize roots.

1. Introduction

Azospirillum brasilense is a soil bacterium capable of colonizing the roots of their plant hosts and of promoting plant growth. The enhancement in plant growth has been mainly attributed to the capability of *A. brasilense* to produce plant growth-promoting substances such as auxins, gibberellins, and cytokinins (Steenhoudt and Vanderleyden 2000). The root colonization by *A. brasilense* has been studied for decades and several bacterial surface components such as polar flagellum (Michiels et al., 1991; Croes et al., 1993), EPS (Michiels et al., 1990; Skvortsov and Ignatov 1998; Burdman et al., 2000; Daniels et al., 2006), and the LPS (Jofré et al., 2004) among others, have been involved. Early colonizing events include the reversible binding of bacteria onto the root surface; a process called adsorption, followed by the irreversible attachment of bacteria to root, named anchoring (Michiels et al., 1991).

It has been demonstrated that adsorption of *A. brasilense* to wheat roots is dependent on the presence of a functional polar flagellum (Michiels et al., 1991). Moreover, purified polar flagella from *A. brasilense* were shown to bind specifically to wheat roots (Croes et al., 1993).

Two flagellar systems are present in *A. brasilense* (Tarrand et al., 1978). A single polar flagellum, constitutively expressed, allows the bacteria to swim in liquid environments, while the induction of lateral flagella synthesis is responsible for swarming on surfaces (Hall and Krieg 1984). Studies on flagellar gene expression of *A. brasilense* Sp7 have shown that the lateral flagella gene, *laf1*, is induced when the polar flagellum is hindered in its rotation (Moens et al., 1995a).

In *A. brasilense* Sp7, a strain very closely related to strain Cd, a large collection of genes encoding proteins, involved in biosynthesis of cell surface polysaccharides and motility, had been previously reported through sequencing and through *in silico* analysis of pRhico plasmid (Vanbleu et al., 2004). Furthermore, defects in flag-

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http://dx.doi.org/10.1016/j.micres.2016.05.006
0944-5013/

ellar assembly, motility and ability to adsorb to plant roots were observed in a mutant of *A. brasilense* Sp7 with a deletion in a region essential for motility (Mot3) present in the pRhico plasmid (Michiels et al., 1991).

Flagellin *O*-glycosylation is a common posttranslational modification present in the flagella of several Gram-negative bacteria such as *Caulobacter crescentus, Aeromonas caviae, Helicobacter pylori, Campylobacter jejuni,* among others (Leclerc et al., 1998; Gryllos et al., 2001; Creuzenet 2004; Merkx-Jacques et al., 2004 Merkx-Jacques et al., 2004). The genes required for flagellin glycosylation are usually grouped in a cluster close to the genes encoding the flagellar apparatus (Merino and Tomás 2014). The *flmA* and *flmB* genes encoding an epimerase/dehydratase and a NAD(P) dependent—aminotransferase, are thought to be implicated in the biosynthesis of the sugar moiety present in flagellin from polar flagellum (Power and Jennings 2003). In *Aeromonas* spp., mutation in *flmB* affected motility, flagellar assembly and adherence (Gryllos et al., 2001).

The flagellin from polar flagellum of *A. brasilense* Sp7 was previously reported to be glycosylated (Moens et al., 1995b) and homologs to *flmA* and *flmB* genes were previously detected in the pRhico plasmid (Vanbleu et al., 2004). Here, we describe two genes of *A. brasilense* Cd, homologs to *flmA* and *flmB*, and its contribution to the bacterial motility, flagellar assembly and attachment to maize roots. We have also studied the effects of mutations in *flmA* or *flmB* on the LPS profile and EPS production.

2. Materials and methods

2.1. Bacterial strains, plasmid and media

The bacterial strains and plasmids used in this study are listed (Table 1). *A. brasilense* was grown at 30 °C in Lysogeny broth (LB) supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ (Miller 1972) or minimal media lactate (MML) (Dreyfus et al., 1983). *Escherichia coli* strains were grown at 37 °C in LB medium. Antibiotics were added, when required, at the following concentrations: Kanamycin (Km), 25 μg ml^{-1}; Tetracycline (Tc), 10 μg ml^{-1}; Nalidixic Acid (NA), 50 μg ml^{-1}, and Chloramphenicol (Cm), 30 μg ml^{-1}.

2.2. DNA manipulations

Plasmid and total DNA preparations, agarose gel electrophoresis, restriction-endonuclease digestion and cloning were performed according to standard protocols (Sambrook et al., 1989). *E. coli* transformation was achieved by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories Ltd.). Southern hybridizations were performed using a non-radioactive probe labeled with 14-dCTP-biotin and a chemiluminescence method was used to detect hybridization bands, according to the instructions of the manufacturer (Gibco BRL, Life Technologies). The IS50 sequences from transposon Tn*5* were amplified by PCR using primers IS1 and IS2 (Table S1) and used as probe.

2.3. Tn*5* Mutagenesis

Random transposon Tn*5* mutagenesis of *A. brasilense* Cd was carried out as described previously (Jofré et al., 2004) by using the mobilizable pGS9 suicide plasmid (Vanstockem et al., 1987). The kanamycin-resistant transconjugants were selected on MML medium.

2.4. Motility assays

Swimming was determined in motility medium (MM) (Tabei et al., 2009) supplemented with 0.3% agar. Swimming plates were point-inoculated by using a toothpick, with bacterial cultures grown till an optical density at 600 nm (OD_{600}) = 0.6. The plates were incubated at 30 °C for 48 h. Bacterial motility was assessed quantitatively by measuring the diameter of the turbid zone formed by the bacterial cells migrating away from the point of inoculation.

2.5. Transmission electron microscopy (TEM)

Bacterial strains were grown at 30 °C and 75 rpm in LB broth supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ till OD_{600} = 0.6. Culture aliquots were placed on carbon-coated grids and stained with 2% solution of potassium phosphotungstate (pH 5.2; 2% w/v KOH). Preparations were observed on a JEM1200 EX II (JEOL, Japan Electron Optics Laboratory Co., Ltd) transmission electron microscope at 80 kV and photographs were taken on a Kodak electron image film.

2.6. Cloning of the Tn*5*-interrupted region

Total DNA from *A. brasilense* CC1 was digested with *Eco*RI and ligated to pSUP102. The ligation mixture was used to transform electrocompetent *E. coli* S17-1 cells. Transformant clones were selected on LB medium containing Km and Tc and screened for the presence of IS50 sequences. This was done with a PCR assay with the primers IS1 and IS2, which reaction leads to a PCR product of 1.2 kb. One of the plasmid isolates (Tc^r, Km^r) was designated as pSC1.

2.7. Subcloning and outward sequencing from the transposon in both directions

Plasmid pSC1 was digested with *Bam*HI-*Eco*RI and the products ligated to pBluescript SK. The recombinant plasmids (pBSK1 and pBSK2) were used to transform electrocompetent *E. coli* JM109 cells. Clones were selected on LB medium containing Ap and Km and LB medium containing Ap and X-gal. Plasmids were isolated, purified, and sequenced using the Taq FS DNA polymerase and the fluorescent-dideoxy terminators in a cycle sequencing method. The resultant DNA fragments were electrophoresed and analyzed using an automated Applied Biosystems 377 DNA sequencer. The amino acid sequences, deduced from the nucleotide sequence, were compared to the GenBank database through the use of the BLAST algorithm (Altschul et al., 1997). The GenBank accession number for the nucleotide sequence determined in this work was KT006319.

2.8. Site-directed mutagenesis by plasmid integration

Internal fragments of the *flmA, flmB,* and *motA* (400, 269, and 573 bp, respectively) were PCR-amplified using the primers flmA-forw and flmA-rev, flmB-forw and flmB-rev and motA-forw and motA-rev, respectively (Table S1). The primers for *flmA* were designed based on the nucleotide sequence of *flmA* from plasmid pRhico GenBank AY523973.1 (Vanbleu et al., 2004). The primers for *motA*, from the nucleotide sequence of *motA* gene, were designed from the *A. brasilense* Sp7 genome (http://img.jgi.doe.gov.). Primers for *flmB* were obtained from the nucleotide sequence obtained for *flmB* of *A. brasilense* Cd (this work, GenBank KT006319). PCR products were purified by means of the QIAquick®/™ PCR-purification kit (QIAGEN), restricted with *Eco*RI/*Bam*HI for *flmA*, *Bam*HI/*Hind*III for *flmB,* and *Eco*RI/*Hind*III for *motA* (restriction sites are underlined in the above primers) and then ligated to the corresponding sites in the pK18mob2 vector.

Table 1
Bacterial strains, plasmids and primers used in this study.

Strains	Relevant characteristics	Reference or source
A. brasilense Cd	ATCC 29710 wild-type.	Tarrand et al. (1978)
A. brasilense CC1	*flmB*:Tn*5* mutant of *A. brasilense*.	This work
A. brasilense CC2	*A. brasilense* nonpolar *flmB* mutant	This work
A. brasilense CC3	*A. brasilense* nonpolar *flmA* mutant	This work
A. brasilense CC4	*A. brasilense* nonpolar *motA* mutant	This work
E. coli DH5α	*endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ (*lacZYA-argF*)*U169* Φ80d *lacZ* Δ*M15*.	Hanahan (1983)
E. coli JM109	*recA endA1 gyrA96 thi hsdR17 supE44* Δ(*lac-proAB*) *relA1*.	Sambrook et al. (1989)
E. coli S17-1	*pro recA hsdR, thi* RP4-2-Tc:Mu-Km:Tn*7* integrated into the chromosome.	Simon et al. (1983)
Plasmids		
pK18mob2	pK18mob derivative with unique *Kpn*I and *Sac*I sites in polylinker, Km^r	Tauch et al. (1998)
pGS9	Cm^r, Km^r, (Tn*5*) p15A replicon N-*tra* Tn*5* donor.	Selvaraj and Iyer (1983)
pSUP102	Tc^r, Cm^r, vector movilizable pACYC184 derivative.	Simon et al. (1989)
pBluescript SK	Cloning and sequencing vector, Ap^r, phagemid, M13 derivative, f1 origin of replication.	Stratagene, La Jolla, California
pFAJ1708	Broad-host-range expression vector, P_{nptII} $oriV^{RK2}$ Tc^r	Dombrecht et al. (2001)
pSC1	12-kb *Eco*RI fragment containing Tn*5* insertion and flanking DNA from *A. brasilense* CC1 cloned into pSUP102; Km^r Tc^r.	This work
pBSK1	8-kb *Eco*RI-*Bam*HI fragment, containing flanking DNA, IS*50*L and *nptII*, from pSC1 cloned into pBluescript SK; Km^r Ap^r.	This work
pBSK2	4-kb *Eco*RI-*Bam*HI fragment containing IS*50*R and flanking DNA, from pSC1, cloned into pBluescript SK; Ap^r lac^-.	This work
pKflmA	400-bp internal fragment of *flmA* from *A. brasilense* cloned into pK18mob2, Km^r.	This work
pKflmB	269-bp fragment containing *flmB* from *A. brasilense* cloned into pK18mob2, Km^r.	This work
pkmotA	573-bp internal fragment of *motA A. brasilense* cloned into pK18mob2, Km^r.	This work
pFAJflmA	pFAJ1708 with *flmA* gene from *A. brasilense* Cd	This work
pFAJflmB	pFAJ1708 with *flmB* gene from *A. brasilense* Cd	This work

All plasmidic constructions were verified by sequencing. The recombinant plasmids pKflmA, pKflmB, and pKmotA were mobilized by mating *E. coli* S17-1 to *A. brasilense* Cd. Transconjugants were selected in MML medium supplemented with Km.

2.9. Plasmid generation for complementation assays

Plasmids pFAJflmA containing the complete coding region for *flmA* (plus 60 bp upstream of the ATG and 45 bp downstream of the TGA) and pFAJflmB containing the complete coding region for *flmB* (plus 118 bp upstream of the ATG and 30 bp downstream of the TGA) were obtained by PCR amplification of *A. brasilense* Cd genomic DNA. This was done using oligonucleotides flmAcompl-FOR and flmAcompl-REV to generate a PCR product of 1188 bp and flmBcompl-FOR and flmBcompl-REV to generate a PCR product of 1399 bp (Table S1). The PCR products were purified, restricted with *Bam*HI/*Eco*RI (for *flmA*) and *Hind*III/*Eco*RI (for *flmB*) and, then, ligated to the corresponding sites in the pFAJ1708 vector. The recombinant plasmids pFAJflmA and pFAJflmB were introduced into *E. coli* S17-1 and then mobilized by mating to *A. brasilense*.

2.10. Flagellin preparation and analysis

Flagellins were extracted from wild-type and mutant cells according to Althabegoiti et al. (2011). *Azospirillum* strains were grown in MML till OD_{600} = 1, vortexed for 5 min and centrifuged at 10000*g* for 30 min at 4 °C. The supernatant was incubated with 1.33% polyethyleneglycol (PEG) and 166 mM NaCl for 3 h at 4 °C, centrifuged at 11000*g* for 40 min at 4 °C and the resulting pellet was suspended in phosphate-buffered saline. Samples were boiled in Laemmli loading buffer for 10 min and then separated by SDS-PAGE and stained with Coomassie brilliant blue by standard methods (Laemmli, 1970).

Glycoproteins were detected by using the commercial kit GlycoProfileTM III (SIGMA) according to the instructions of the manufacturer. Visualization of fluorescent bands was achieved by using an UV transiluminator coupled to a digital camera (Alpha Innotech).

The identity of flagellins was confirmed by immunoblotting analysis, employing a polyclonal antibody raised against the polar flagellum of *A. brasilense* Sp7 (Hall and Krieg, 1984). Purified flagellar proteins were separated by SDS-PAGE and transferred onto PVDF membranes for immunoblotting in a semidry electrophoretic transfer cell, as described by Towbin et al. (1979). The flagellin from polar flagellum was detected with an anti-polar flagellum antibody. The primary antibody was diluted in Tris-buffered saline supplemented with 0.1% (vol/vol) Tween 20 and 0.3% (wt/vol) non-fat dry milk. The binding of the secondary antibody, the anti-rabbit immunoglobulin G peroxidase conjugate (Sigma), was detected by ECL chemiluminescence reagents (Thermo Scientific).

2.11. Competition for attachment to maize roots

Seeds of *Zea mays* cv. 'NK910 TDMax' were surface-sterilized and germinated as described previously (Jofré et al., 1996). Three-day-old maize seedlings were inoculated with 4 ml of Hoagland (Hoagland and Arnon 1950) medium containing 1×10^6 CFU of each competing strain of *A. brasilense*. After 2 h of incubation at 30 °C and 150 rpm (for assays with shaking), roots were washed twice by immersion in sterile 0.88% NaCl and then macerated in sterile 0.88% NaCl. The suspension was used to determine the number of

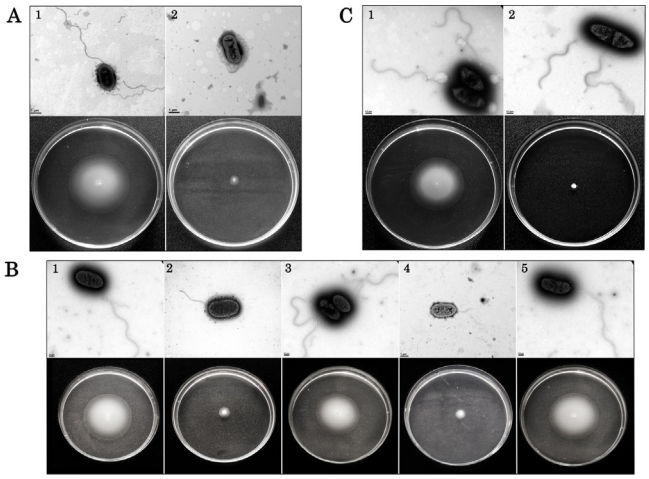

Fig. 1. (A) TEM (up) and swimming (down) images of *A. brasilense* Cd (1) and the mutant strain CC1 (Tn*5* mutant) (2). Bar = 1 μm.
(B) TEM (up) and swimming (down) images of *A. brasilense* Cd (wild type) and non-polar flmA and flmB mutants. Bars = 0.5 μm (1, 3, 5) or 1 μm (2, 4).
1: *A. brasilense* Cd; 2: *A. brasilense* CC3 (flmA mutant); 3: *A. brasilense* CC3 + pFAJflmA; 4: *A. brasilense* CC2 (flmB mutant); 5: *A. brasilense* CC2 + pFAJflmB.
(C) TEM (up) and swimming (down) images of *A. brasilense* Cd wild type (1) and motA mutant (2). Bar = 0.5 μm.
In all cases, swimming phenotype was evaluated in motility medium (0.3% agar). For TEM, strains were grown in LB broth till OD_{600} = 0.6.

attached bacteria by plate counting in MML medium supplemented or not with the appropriate antibiotics.

2.12. EPS isolation and quantification

For EPS isolation, *A. brasilense* strains were grown in MML containing 10 mM NH_4Cl at 30 °C for 2 days. EPS were precipitated from culture supernatants with three volumes of cold ethanol. Pellets were air-dried, suspended in distilled water and dialyzed (MWCO 12 000 Da) against distilled water for 2 days at 4 °C. EPS were quantified by using the anthrone method (Dische 1962).

2.13. LPS isolation and analysis

LPS was purified from *A. brasilense* cells grown in LB broth by the method of Westphal and Jann (1965). LPS was separated by 20% (w/v) polyacrylamide-gel electrophoresis (PAGE) in an SDS-Tricine buffer system and visualized by silver staining (Tsai and Frasch 1982).

2.14. Statistical analysis

Data were analyzed by one way analysis of variance (ANOVA) or the Student test when appropriate. Differences were considered to be significant at the $P < 0.05$ level. The means were evaluated for significance through the Bonferroni test.

3. Results

A collection of Tn*5* mutants of *A. brasilense* Cd was generated as described previously (Jofré et al., 2004), and screened for alterations in motility on swimming agar plates. Mutant strain CC1, among the 2700 colonies screened, showed a significant reduction in swimming motility compared to that of the wild-type strain (Fig. 1A). Transmission electronic microscopy (TEM) of cells of the CC1 strain grown in LB broth, till the exponential phase growth demonstrated the presence of a severely truncated polar flagellum, thus suggesting that Tn*5* insertion affected

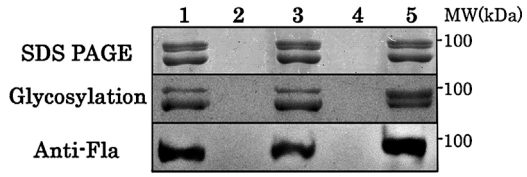

Fig. 2. Analysis of flagellar proteins of *A. brasilense* wild type and *flmA* and *flmB* mutants.
SDS-PAGE of flagellar extracts (SDS-PAGE), glycoprotein detection (Glycosylation) and immunowesternblot (anti-Fla).
1: *A. brasilense* Cd (wild type); 2: CC3 (*flmA* mutant); 3: CC3pFAJflmA; 4: CC2 (*flmB* mutant)and 5: CC2pFAJflmB.

the expression of a gene required for polar flagellum synthesis. In contrast, TEM observations of wild type cells demonstrated the presence of a single polar flagellum (Fig. 1A). Lateral flagella were not observed either in the cells of the mutant strain or in the cells of the wild type strain. Southern blot analysis, using a specific probe for the Tn*5* transposon, showed that the genome of the mutant strain CC1 contains a single Tn*5* insertion (data not shown). An *Eco*RI DNA fragment containing the transposon Tn*5*, present in the genome of mutant CC1, was first cloned into plasmid pSUP102 and then subcloned, as *Eco*RI/*Bam*HI fragments, into pBluescript SK and sequenced (accession number KT006319). The obtained nucleotide sequence was analysed with the aid of the ORF-finder software (available in the GenBank database) and the BLAST algorithm to compare nucleotide sequences and their translation products against the GenBank database. The DNA fragment flanking the Tn*5* insertion, in the genome of mutant strain CC1, encoded a product with 100% of identity and similarity to the FlmB protein of *A. brasilense* involved in flagellin modification. FlmB proteins are homologs of the PseC family proteins (UDP-4-amino-4,6-dideoxy-*N*-acetyl-beta-L-altrosamine transaminase family). This family of enzymes are aminotransferases of the pfam01041 family involved in the biosynthesis of pseudaminic acid. They convert UDP-4-keto-6-deoxy-*N*-acetylglucosamine into UDP-4-amino-4,6-dideoxy-*N*-acetylgalactose. Pseudaminic acid has a role in surface polysaccharides in *Pseudomonas* (Knirel et al., 1984), as well as in the modification of flagellin in the species of *Campylobacter* (Thibault et al., 2001; Schirm et al., 2005; Logan et al., 2009); *Aeromonas* (Tabei et al., 2009) and *Helicobacter* (Schirm et al., 2003, 2005).

In the three genomes of *A. brasilense* available, up to now, the *flmB* gene forms a dicistronic operon with *flmA*. Both genes are flanked by the tRNA_Gly, a hypothetical protein, *rkpK* (UDP-glucose dehydrogenase) and *exoC* (phosphoglucomutase) genes (Fig. S1). In contrast to the genetic arrangement observed in most bacteria, in *A. brasilense*, the flagella structural genes were not detected as being close to the *flmAB* genes.

3.1. Non-polar mutations in flmA or flmB result in a truncated polar flagellum and impaired swimming motility

Since Tn*5* insertions often result in polar effects on the transcription of downstream genes, we decided to generate non-polar mutants in *flmA* and *flmB* genes from *A. brasilense* Cd and study their phenotypes.

Non-polar mutants were generated by targeted insertion of an integrative plasmid (see Section 2). In these mutants, the *lac* promoter of the integrated plasmid (pKflmA or pKflmB) directs transcription of the downstream gene.

Both mutant strains, designed as *A. brasilense* CC2 (pKflmB integrated into *flmB*) and CC3 (pKflmA integrated into *flmA*), showed a drastic reduction in swimming motility on semi-solid media

(0.3% w/v agar) in comparison to the wild type strain (Fig. 1B). In agreement with the observed phenotype for the mutant strain CC1 (Tn*5* insertion mutant), cells from both mutant strains CC2 and CC3 grown in liquid media and examined by TEM showed a severely truncated polar flagellum. This indicated that the reduced motility is due to alterations in the flagellar structure. In contrast, the wild type strain showed a long polar flagellum. These data suggest that FlmA and FlmB proteins from *A. brasilense* Cd influence the assembly and, consequently, the functioning of the polar flagellum. The mutant strains CC2 and CC3 complemented in *trans* with their corresponding wild type alleles (CC2-pFAJ*flmB* and CC3-pFAJ*flmA*) regained the phenotypes observed in the wild-type strain (Fig. 1B).

No differences were observed in the growth of the mutant strains CC2 and CC3, compared to the wild type strain (data not shown).

Moens et al. (1995b) reported that flagellin from polar flagellum of *A. brasilense* Sp7 is glycosylated, however, genes involved in the glycosylation process have not been identified yet. SDS-PAGE of flagellar extracts from the wild type and complemented strains CC3-pFAJflmA and CC2-pFAJflmB showed the presence of three bands of approximately 100 kDa (Fig. 2). These bands gave a strong signal when probed by immunoblotting with an *A. brasilense* anti-polar flagellum antibody (Anti-Fla) (Hall and Krieg 1984). This result confirmed that the bands observed in the SDS-PAGE of flagellar extracts corresponded to the flagellin from the polar flagellum of *A. brasilense*. It is important to highlight that we did not achieve the separation in three bands in the immunoblot and glycosylation gels observed in the SDS-PAGE. As expected, no flagellins were detected in the purified extracts from mutant strains CC3 (*flmA*) and CC2 (*flmB*) (Fig. 2(2) and (4) respectively). The wild-type phenotype was restored in the complemented strains CC3-pFAJflmA and CC2-pFAJflmB (Fig. 2(3) and (5). Furthermore, purified flagellins from wild-type and complemented strains were glycosylated (Fig. 2). In agreement with the absence of flagellin, no signal for glycosylation was observed in flagellar extracts from mutant strains CC3 and CC2.

The differences in size between the three flagellin bands could be due to different extents of glycosylation.

Our data suggest the participation of the *flmAB* operon in the glycosylation and/or assembly of the polar flagellum of *A. brasilense* Cd.

3.2. LPS profile and EPS production

Belyakov et al. (2012) reported that polar flagellum flagellin of *A. brasilense* Sp7 is glycosylated with a branched tetrasaccharide repeating unit similar to that observed in the *O*-polysaccharide chain of the LPS of the same strain. Those authors have suggested that the genes responsible for *O*-polysaccharide biosynthesis of the LPS are the same involved in the synthesis of the saccharide moiety present in glycosylated flagellin. To assess whether *flmA* and *flmB* were involved in LPS biosynthesis in *A. brasilense* Cd, the LPS pattern of mutant strains CC2 and CC3 were analyzed by SDS-PAGE. Silver stained gels revealed changes in the electrophoretic pattern of the LPS from *flmB* and *flmA* mutants (CC2 and CC3 strains, respectively) compared to that of the wild type strain. LPS from mutant strains CC2 and CC3 showed an extra band between the LPS I (smooth LPS) and LPS II (rough LPS) bands. This extra band was missing in the LPS from the wild type strain (Fig. 3A). Both mutant strains complemented with their corresponding wild type alleles showed a similar LPS pattern as the one observed for the wild type strain, thus indicating that the observed phenotypes were the consequence of the inactivation of *flmA* and *flmB* genes.

We also determined whether mutations in *flmA* and *flmB* affected the production of EPS in *A. brasilense* (Fig. 3B). For that, supernatants of cultures grown for 48 h in MML medium from the wild type and mutant strains, were used to obtain EPS. Both mutant strains CC2 and CC3 produced almost twice more EPS than the wild type under the same conditions. These differences in EPS content were not observed when the mutant strains were complemented with their corresponding wild type alleles (Fig. 3B).

3.3. Competition for adsorption to maize roots is diminished in mutants affected in either flmA or flmB

The polar flagellum of *A. brasilense* plays an important role in the adsorption to plant roots (Croes et al., 1993). Because of this, the competitive fitness for attachment to maize roots of each mutant strain, CC2 and CC3, was compared to that of the wild type strain. For that, the assay was performed co-inoculating maize roots with the same number of cells from the wild type strain and the mutant strains. Under no shaking conditions, both mutant strains showed a significantly reduced adsorption to maize roots in competence with the wild type strain (Table 2). In contrast, the wild type strain and the complemented mutant strains CC2-pFAJ*flmB* or CC3-pFAJ*flmA* competed equally for adsorption to maize roots.

We asked whether this reduced attachment to maize roots observed in the *flmA* and *flmB* mutants was the consequence of the lack of motility or the absence of flagellin from the polar flagellum (acting as an adhesion protein). Therefore, we performed the same attachment assay under shaking conditions. We hypothesized that under these conditions the probability to achieve root binding sites for wild type and mutant strains is almost the same. It is noticeable that, under shaking conditions, the level of bacterial attachment was 10 times higher than that observed without shaking. Under shaking conditions however, both *A. brasilense* mutants in *flmA* or *flmB* were less competitive than the wild type strain in their adsorption to maize roots (Table 2). Complementation of the mutant strains with their corresponding wild type alleles restored the competitiveness to values observed for the wild type strain. We performed the same assay using different ratios of the competitor strains. Under shaking conditions, co-inoculating the wild type strain and the mutant strain CC2 or CC3 at a ratio of 1–0.1, the number of cells of the mutant strains attached to the roots was drastically reduced (almost 5 and 10 times less than the observed, co-inoculating the same number of cells of the wild type and mutant strains). In the opposite situation, co-inoculating the wild type strain and the CC2 or CC3 mutant at a ratio of 0.1–1, the percentages of adsorption of the mutant strains were similar to the ones observed for the wild type strain. Under no shaking conditions, no attachment was detected for the mutant strains when co-inoculated with the wild type strain at the ratio of 0.1–1. When the wild type strain was co-inoculated with the mutant strain CC2 or CC3 at a ratio of 0.1–1, the level of adsorption to maize roots observed for both mutant strains was comparable to that of the wild type strain (Table 2).

3.4. Motility contributes to the attachment of A. brasilense Cd to maize roots

To determine whether motility or the polar flagellum as a root binding protein is responsible for attachment to maize roots, a mutant strain expressing the polar flagellum affected in its rotation was generated by targeted insertion of an integrative plasmid. In this mutant strain, named CC4, the *motA* gene, coding for a subunit of the protein complex that form the ion channel responsible for torque generation (Belas 2014), was interrupted. Swimming motility and the production of polar flagellum were determined in the mutant strain CC4 and compared with those of the wild type strain (Fig. 1C). As shown in Fig. 1C, the mutant strain CC4 was non-motile in swimming agar but still produced a complete polar flagellum.

The ability of this mutant strain to attach to maize roots was evaluated under shaking and no shaking conditions, in competi-

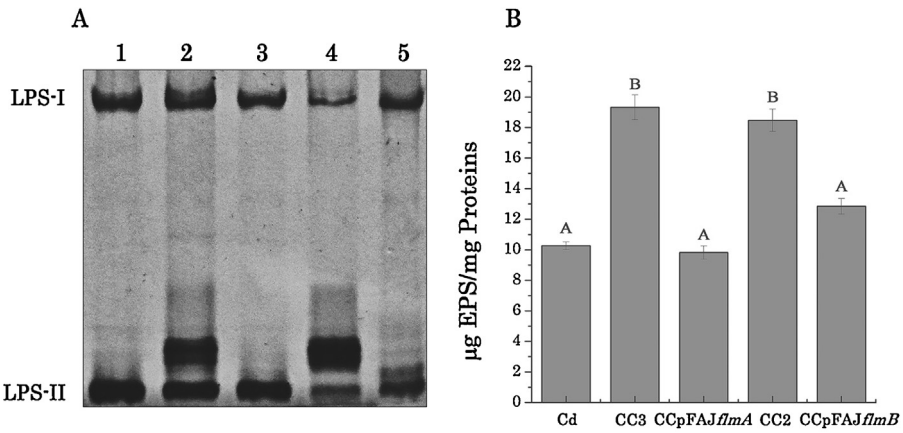

Fig. 3. Analysis of LPS (A) and EPS (B) produced by *A. brasilense flmA* and *flmB* mutants.
(A) SDS-PAGE separation and silver stain detection of LPS extracted from *A. brasilense* Cd (wild type) and *flmAB* mutants.
(B) Data are the means ± SEM of three independent experiments with three replicates each. Statistical analyses were done by one-way analysis of variance. Different letters indicate a significant difference ($P \leq 0.01$).
A and B, Cd and lane 1: *A. brasilense* Cd (wild type); CC3 and lane 2: *A. brasilense* CC3 (*flmA* mutant); CCpFAJflmA and lane 3: *A. brasilense* CC3 + pFAJflmA; CC2 and lane 4: *A. brasilense* CC2 (*flmB* mutant); CCpFAJflmB and lane 5: *A. brasilense* CC2 + pFAJflmB.

Table 2
Competition for adsorption to maize roots between the *A. brasilense* wild type and *flmA* or *flmB* mutants.

Competing strains/ratio	Adsorption to maize roots			
	With shaking		Without shaking	
	Number of adsorbed bacteria (CFU × 10^4/root[a])	Percentage of adsorption of each strain/root[b]	Number of adsorbed bacteria (CFU × 10^3/root[a])	Percentage of adsorption of each strain/root[b]
WT vs CC3 1: 1	$7.00^A \pm 1.13$ vs $1.60^B \pm 0.45$	81 vs 19	$6.30^A \pm 1.04$ vs $1.10^B \pm 0.08$	85 vs 15
WT vs CC3 1: 0.1	$7.25^A \pm 1.27$ vs $0.14^B \pm 0.03$	98 vs 2	$4.40^A \pm 0.85$ vs nd[c] (<0.3) ^{B}	
WT vs CC3 0.1: 1	$2.15^A \pm 1.12$ vs $3.05^A \pm 0.97$	41 vs 59	$3.05^A \pm 1.27$ vs $1.40^A \pm 0.21$	68 vs 32
WT vs CC3-pFAJflmA 1: 1	$4.10^A \pm 0.57$ vs $4.86^A \pm 1.29$	46 vs 54	$4.40^A \pm 0.63$ vs $4.90^A \pm 1.42$	47 vs 53
WT vs CC2 1: 1	$4.83^A \pm 0.41$ vs $1.36^B \pm 0.38$	78 vs 22	$6.20^A \pm 1.51$ vs $0.86^B \pm 0.12$	88 vs 12
WT vs CC2 1: 0.1	$6.50^A \pm 1.18$ vs $0.28^B \pm 0.04$	96 vs 4	$5.77^A \pm 0.94$ vs nd[c] (<0.3) ^{B}	
WT vs CC2 0.1: 1	$3.15^A \pm 0.76$ vs $5.62^A \pm 1.11$	36 vs 64	$2.71^A \pm 0.96$ vs $1.47^A \pm 0.09$	65 vs 35
WT vs CC2-pFAJflmB 1: 1	$4.91^A \pm 0.28$ vs $3.95^A \pm 0.45$	55 vs 45	$5.03^A \pm 0.20$ vs $4.41^A \pm 0.42$	53 vs 47
WT vs CC4 1: 1	$7.85^A \pm 1.45$ vs $1.15^B \pm 0.608$	87 vs 13	$5.44^A \pm 1.62$ vs nd[c] (<0.3)^B	
WT vs CC4 1: 0.1	$6.08^A \pm 1.18$ vs $0.095^B \pm 0.039$	98.5 vs 1.5	$7.41^A \pm 1.7$ vs nd[c] (<0.3)^B	
WT vs CC4 0.1: 1	$4.13^A \pm 1.22$ vs $2.39^A \pm 0.73$	63 vs 37	$3.57^A \pm 0.69$ vs nd[c] (<0.3)^B	

[a] Data are the means ± SEM of three independent experiments with 5 replicates each. Statistical analyses were done by the Student's *t*-test. Different letters indicate a significant difference ($P \leq 0.05$).
[b] The level of attachment of both competing strains was taken as 100%.
[c] nd: Not detected (Values below the detection limit of the method for counting CFU).

tion with the wild type strain at different ratios of co-inoculation (1–1, 1–0.1 and 0.1–1 of the wild type strain and mutant strain CC4, respectively). Under shaking conditions, the co-inoculation of both strains at the same number of cells (1–1) showed that the mutant strain CC4 was less competitive than the wild type strain. Only 13% of the root binding sites were occupied by the CC4 strain, whereas the remaining 87% were occupied by the wild type strain (Table 2). The co-inoculation of the wild type strain and mutant strain at a ratio of 1–0.1 resulted in a very low adsorption of the mutant strain to maize roots. When the wild type strain and the mutant strain were co-inoculated at the ratio of 0.1–1, similar percentages of adsorption for both competing strains were observed. Under no shaking conditions, the mutant strain CC4 was not detected at any co-inoculation ratio (Table 2).

Altogether, these data suggest that motility could be a significant trait for the early colonization of *A. brasilense* Cd to maize roots.

4. Discussion

In this study, we characterized a Tn*5* mutant of *A. brasilense* Cd affected in its swimming motility. This phenotype was associated to the production of a truncated polar flagellum. Genetic analysis revealed that transposon Tn*5* was inserted into an ORF encoding for a protein with a significant similarity to PseC/FlmB proteins. These proteins, named PseC in *Campylobacter* and *Helicobacter* (Schoenhofen et al., 2006b) or FlmB in *Caulobacter* and *Aeromonas* (Leclerc et al., 1998; Gryllos et al., 2001), are NAD(P) dependent—aminotransferases involved in flagellin modification. The *pseC*/*flmB* genes are grouped in a cluster together with the *pseB*/*flmA* genes, which products, PseB/FlmA, are epimerases/dehydratases also involved in flagellin modification. These proteins, PseB/PseC of *C.jejuni* (Creuzenet 2004; McNally et al., 2006; Morrison et al., 2008 McNally et al., 2006; Morrison et al., 2008) and *H. pylori* (Creuzenet et al., 2000; Schirm et al., 2003; Schoenhofen et al., 2006a) or FlmA/FlmB of *Aeromonas caviae* Sch3 N (Gryllos et al., 2001; Tabei et al., 2009) are essential during the early stages of the biosynthesis of a sialic acid-derivativenamed pseudaminic acid (Pse5Ac7Ac). Pse5Ac7Ac has been found in many Gram-negative bacterial species, as constituents of cell surface glycoconjugates such as LPS (Knirel et al., 2003), capsular polysaccharide (Kiss et al., 2001), and flagella (Thibault et al., 2001; Logan et al., 2002, 2009; Schirm et al., 2003, 2005; Tabei et al., 2009).

Non-polar mutations in the *A. brasilense* Cd *flmA* or *flmB* genes resulted in the production of a short nonfunctional polar flagellum with the consequently non swimming phenotype, suggesting that the products of *flmAB* genes were required for polymerization and/or export of the flagellin from polar flagellum.

The genomic organization of the *flmA-flmB* cluster presented perfect synteny in the *A. brasilense* Sp7, Sp245, and Az39 strains. The *rkpK* and *exoC* genes (encoding UDP glucose dehydrogenase and phosphoglucomutase, respectively) which products are associated to the EPS/LPS biosynthesis, were detected as adjacent to *flmAB*.

In *A. caviae* Sch3N, the *flmAB* genes also localized within the LPS *O*-antigen biosynthetic cluster (Gryllos et al., 2001). In contrast to the genetic arrangement presented above, in *Campylobacter,* these genes were localized within a hypervariable region containing the structural flagellin genes *flaA* and *flaB* (Dorrell et al., 2001; Pearson et al., 2003; Fouts et al., 2005).

Considering the particular genetic arrangement observed for *flmAB* in *A. brasilense*, the changes detected in the LPS profile and EPS production of the mutant strains suggest that the *flmAB* gene products were shared by the polar flagellum and polysaccharide biosynthetic pathways. A similar phenotype was reported in *A. caviae* Sch3N, where a mutation in *flmA* or *flmB* affected both, flagellar assembly and LPS *O*-antigen synthesis (Gryllos et al., 2001; Tabei et al., 2009). The observed changes were attributed to alterations in the glycosylation pattern of flagellins and LPS *O*-antigen. Thus, in *A. caviae*, the *flmAB* genes form part of a cluster involved in the synthesis of Pse5Ac7Ac which is present in both, flagellins and LPS.

The glycosylation of the flagellin monomer composing the polar flagellum of *A. brasilense* Sp7 had been early demonstrated (Moens et al., 1995b).

Presumably, the lack of the polar flagellum in *flmA* and *flmB* mutants was due to an incorrect assembly or export of the flagellin subunits. Studies in *A. caviae* indicated that unglycosylated flagellins were not exported and, consequently, did not form functional filaments (Parker et al., 2014).

A close similarity between the glycans present in the flagellin from polar flagellum and in the LPS of *A. brasilense* Sp7 has been suggested. Belyakov et al. (2012) reported that the structure of the *O*-glycan modifying the flagellin, is a 7.7-kDa oligosaccharide with the same monosaccharide residue ratio as that observed in the *O*-antigen LPS chain of this strain. These data together with our observation, that mutations in *flmA* or *flmB* modifies LPS profile in *A. brasilense* Cd, support the hypothesis that *flmAB* form part of a cluster involved in the synthesis of a glycan present in both, flagellin and LPS. Whether this glycan is Pse5Ac7Ac or not remains to be determined.

In addition to the changes in the glycosylation pattern and flagellar assembly, alterations in the adherence and colonization of host cells have also been reported in *Helicobacter*, *Campylobacter* and *Aeromonas flmAB* mutants (Gryllos et al., 2001; Rabaan et al., 2001; Thibault et al., 2001; Schirm et al., 2003; Guerry et al., 2006; Schoenhofen et al., 2006a). It has been previously reported that polar flagellum of *A. brasilense* Sp7 is required for bacterial adsorption to wheat roots. Moreover, it was demonstrated that purified polar flagellum from *A. brasilense* Sp7 binds to wheat roots (Croes et al., 1993). Altogether, these data suggested a role for flagellin as a root binding protein. However, no clear evidence is available whether the motility or the presence of polar flagellum as an adhesin is required for adsorption to plant roots. Here, we also showed that a mutation in *flmA* or *flmB* affected the competitive ability of *A. brasilense* Cd for adsorption to maize roots. In fact, mutants affected in *flmA* or *flmB* were less competitive in the adsorption to maize roots than the wild type strain. Furthermore, a *motA* mutant strain, producing a complete polar flagellum but unable to swim, was less competitive than the wild type strain for the adsorption to maize roots.

In conclusion, the data presented here provide evidence about the role of the *flmAB* gene products in flagellar assembly and polysaccharide production in *Azospirillum brasilense* Cd. Furthermore, we present evidence implying that the same gene products are shared for both, polar flagella and LPS biosynthesis. Our data also suggest that motility contributes to the bacterial adsorption to maize roots.

Acknowledgements

This work was founded by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina and the Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto, Argentina. EJ, MP and DBM are members of the Research Career from CONICET. FAR and JPL are scholarship recipient's from CONICET.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2016.05.006.

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